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1 **Oxidative stress and mitochondrial responses to stress exposure suggest that**
2 **king penguins are naturally equipped to resist stress**

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24

25 **Abstract**

26 Exposure to unpredictable environmental stressors could influence animal health and
27 fitness by inducing oxidative stress, potentially through downstream effects of glucocorticoid
28 stress hormones (*e.g.* corticosterone) on mitochondrial function. Yet, it remains unclear
29 whether species that have evolved in stochastic and challenging environments may present
30 adaptations to alleviate the effects of stress exposure on oxidative stress. We tested this
31 hypothesis in wild king penguins by investigating mitochondrial and oxidative stress
32 responses to acute restraint-stress, and their relationships with baseline (potentially
33 mirroring exposure to chronic stress) and stress-induced increase in corticosterone levels.
34 Acute restraint-stress did not significantly influence mitochondrial function. However, acute
35 restraint-stress led to a significant increase in endogenous antioxidant defences, while
36 oxidative damage levels were mostly not affected or even decreased. High baseline
37 corticosterone levels were associated with an up-regulation of the glutathione antioxidant
38 system and a decrease in mitochondrial efficiency. Both processes might contribute to
39 prevent oxidative damage, potentially explaining the negative relationship observed
40 between baseline corticosterone and plasma oxidative damage to proteins. While stress
41 exposure can represent an oxidative challenge for animals, protective mechanisms like up-
42 regulating antioxidant defences and decreasing mitochondrial efficiency seem to occur in
43 king penguins, allowing them to cope with their stochastic and challenging environment.

44

45

45 **Introduction**

46 Vertebrates respond to stressful and unpredictable environmental stimuli (*e.g.* food
47 shortage, predation, adverse weather conditions, etc.) by activating a set of physiological and
48 behavioural responses defined as a whole as the 'stress response' ¹. One key component of
49 the stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis,
50 ultimately leading to the release of glucocorticoid (GC) hormones in the blood stream. GCs are
51 a group of steroid hormones, central to the regulation of energy balance in vertebrates ^{1,2}. At
52 baseline levels they are involved in the regulation of energy balance associated with both the
53 predictable demands of life-history cycles such as seasonal and diurnal variations ^{2,3}, and the
54 prolonged exposure to environmental stressors ^{4,5}. In contrast, acute increases in GC allow
55 animals to maintain homeostasis when faced with unpredictable environmental events (*e.g.*
56 predation, detrimental weather^{1,6}). A key feature of GCs is their role in redirecting energy
57 allocation from non-essential to essential functions, by regulating energy intake, processing
58 and expenditure ².

59 Elevated baseline GC levels may reflect adaptive responses (*e.g.* stress responsiveness)
60 of individual to cope with specific life-history stages or challenges, but may also reflect
61 situations of chronic stress when organisms are continuously exposed to stressful stimuli that
62 may ultimately affect their fitness ⁷. However, the relationship between baseline GC levels and
63 fitness is not consistent ⁸, and little is known about the physiological pathways relating GCs to
64 individual fitness. Recently, oxidative stress has been suggested as one important down-
65 stream consequence of chronically elevated baseline GC levels, with potential consequences
66 on fitness ⁹⁻¹¹. Oxidative stress is a complex, multi-faceted state that arises in organisms as a
67 consequence of the imbalance between the production of pro-oxidant molecules and
68 antioxidant defences ^{12,13}. Reactive oxygen species (ROS) are one main type of pro-oxidants,

69 and partly arise as by-products of cellular respiration in mitochondria ¹⁴. Although ROS have
70 important roles in cell signalling, they act as a double-edged sword damaging macromolecules,
71 cell components and structures when produced in excess ¹²⁻¹⁴. In the past decade several
72 studies have highlighted direct links between chronic stress exposure and/or chronically
73 elevated baseline GC levels and oxidative stress ^{9,15-17}. There is also growing evidence that
74 acute stress exposure could cause oxidative stress. Accordingly, it has been shown that acute
75 restraint-stress (≤ 1 hour) could increase ROS production ^{18,19}, oxidative damage to lipids and
76 DNA ²⁰⁻²², while having mixed effects on antioxidant defences ¹⁸⁻²⁴. Most of those studies were
77 however conducted in laboratory models adapted to constant and unchallenging
78 environments (*i.e.* food ad-libitum, no predation risk, controlled microclimate, etc.). Hence, we
79 still know little on the effects of stress and the GC response on oxidative stress in the wild. In
80 particular, whether species that evolved in stochastic and challenging environments may
81 present adaptations to cope with stress-induced oxidative stress is unknown.

82 Since mitochondria are the site of aerobic respiration and one major sources of ROS,
83 they have been suggested to play a key role in the stress response ^{25,26}. This idea has however
84 received limited attention so far, even though mitochondrial dysfunction may constitute a
85 central pathway linking stress exposure to impaired organismal maintenance ²⁷. The presence
86 of GC receptors in mitochondria suggests that they may play a role in the stress response ²⁸.
87 GCs are known to affect mitochondrial gene expression, mitochondrial biogenesis, and
88 mitochondrial fission/fusion dynamics, influencing both ATP and ROS production ^{25,26}.
89 However, the way GCs affect mitochondrial function could differ depending on GC dose and
90 duration of exposure (*e.g.* differences between acute and chronic GCs exposure ^{25,29}). For
91 instance, exposure to moderate or high GC levels increases mitochondrial oxidative capacity in
92 the short-term, but prolonged exposure to high GC levels decreases mitochondrial oxidative

93 capacity²⁹. Additionally, GCs have been shown to increase potentially both mitochondrial and
94 non-mitochondrial sources of ROS production³⁰. While we ignore the fine time scale (*i.e.*
95 seconds, minutes, hours) at which GCs could affect mitochondrial function *in vivo*, other
96 hormones such as thyroid hormones have been shown to have very rapid effects (< 1 hour) on
97 mitochondrial respiration³¹. Therefore, rapid changes in mitochondrial function could be part
98 of the acute stress response. Our current knowledge is mostly based on studies using cultured
99 cells, and we lack information on the links between mitochondrial function and GC stress
100 responses at the organism level, particularly in species evolving in their natural environment.
101 The sensitivity of mitochondria to GCs could vary between strains of the same captive species
102³². Thus, it is possible that the relationships between GCs, mitochondrial function, and
103 oxidative stress (see above) could differ between species, life history stages, and
104 environmental conditions.

105 We tested this hypothesis in freely-breeding king penguins (*Aptenodytes*
106 *patagonicus*) by investigating mitochondrial and oxidative stress responses to acute
107 restraint-stress, and their relationships with baseline (potentially mirroring exposure to
108 chronic stress) and stress-induced increase in corticosterone levels. King penguins are an
109 interesting model to investigate the oxidative and mitochondrial responses to stress exposure.
110 These sub-Antarctic seabirds breed on-land while being exposed to numerous environmental
111 stressors (predation, parasites, detrimental weather, aggressive social environment³³⁻³⁵). They
112 have evolved both behavioural and physiological adaptations to tolerate periods of long-term
113 fasting³⁶ (including mitochondrial adaptations³⁷ and adaptations to oxidative stress³⁸). Finally,
114 our studies show that baseline corticosterone (CORT) levels in this species are positively
115 associated with chronic stressors such as high intra-specific density in an aggressive social
116 environment³⁹ and ectoparasites prevalence (Bize et *al.*, unpublished).

117 Here, we subjected 24 free-living incubating king penguins to an acute restraint-stress
118 for 30 minutes. We collected blood samples both before (baseline < 4min post-capture⁴⁰) and
119 at the end of the 30 minutes of restraint-stress. We used these samples to measure baseline
120 and stress-induced plasma total CORT levels and oxidative stress markers measured both in
121 plasma and red blood cells (RBCs). Birds also have functional mitochondria in their RBCs^{41,42},
122 thus allowing us to assess short-term changes in mitochondrial function by repeated blood
123 sampling⁴³. First, we investigated the impact of acute restraint-stress on oxidative stress levels
124 and mitochondrial function. Second, we tested if between-individual differences in CORT
125 response (*i.e.* Δ CORT: T30 – baseline) could predict between-individual differences in oxidative
126 stress and mitochondrial responses to restraint-stress. Indeed, if the acute rise in GCs is the
127 mechanism leading to changes in mitochondrial function and oxidative stress during acute
128 stress, we would expect that birds having a higher CORT response would also show more
129 pronounced changes in oxidative stress levels and mitochondrial function. Finally, we assessed
130 the relationships between baseline CORT levels (potentially mirroring exposure to chronic
131 stress) and both baseline oxidative stress levels and mitochondrial function.

132 Given the numerous environmental challenges faced by king penguins while breeding
133 on land and foraging at sea, we hypothesized that this bird species should have evolved
134 specific adaptations alleviating the effects of stress exposure and/or high GC levels on
135 oxidative stress.

136

137 **Material and Methods**

138 **Study species and sampling procedures**

139 This study took place on Possession Island in the Crozet Archipelago (46° 25'S; 51° 52'E). Data
140 were collected during the 2013-2014 breeding season in the king penguin colony of “La

141 Grande Manchotière" (*ca.* 24,000 breeding pairs). This study has been approved by the ethical
142 committee 'Comité d'éthique de la Fédération de Recherche en Biologie de Toulouse' (C2EA-
143 01 FRBT), project N°119-2013, and all experiments were performed in accordance with
144 relevant guidelines and regulations. During the breeding season, male and female king
145 penguin alternate between periods on-land caring for the egg or chick, and periods foraging at
146 sea ⁴⁴. After a courtship period of *ca.* 15-days on land, the female lays a single egg and the
147 male takes care of the first incubation shift while the female leaves to forage at sea. She
148 returns to relieve her partner *ca.* 15-days later. Alternated guards continue throughout
149 incubation (53 days) and early chick brooding. In this study, we caught females (N = 12) and
150 males (N = 12) from independent breeding pairs at the beginning of their incubation shift.
151 Females were caught at the start of their first incubation shift, and males were caught at the
152 start of their second incubation shift to ensure both sexes had experienced similar fasting
153 durations (birds were all caught 3 days after their return from sea). This was particularly
154 important given the known effects of long-term fasting on both baseline CORT and oxidative
155 stress levels in this species ^{38,45}.

156 Birds caught during incubation were rapidly blood-sampled after capture (< 4min;
157 mean \pm SE = 3'22" \pm 11") after placing the egg in an insulated box and replacing it by a dummy
158 to avoid accidental breakage during handling. Blood (*ca.* 1mL) was taken from the flipper vein
159 using a heparinized syringe. In the king penguin, no detectable increase in baseline GC levels
160 occurs within the first 5 minutes of capture ⁴⁰. Thus, corticosterone levels measured in this first
161 blood sample represent bird's baseline levels under natural conditions. Birds were manually
162 restrained by the same experimenter (EL) and a second blood sample (*ca.* 1mL) was taken *ca.*
163 30 min post-capture (mean \pm SE = 31'22" \pm 14"). All birds continued to incubate at the end of
164 the restraint-stress period, and our manipulations never resulted in breeding failure. All blood

165 samples were kept on crushed ice until centrifugation (30-60 min later) at 3000 *g* for 10 min to
166 separate plasma from RBCs. The plasma fraction was then removed and plasma aliquots were
167 stored at -20°C until the end of the day, before being transferred to -80°C until laboratory
168 analyses. Two aliquots of 100µL of pelleted RBCs were transferred into a new tube containing
169 1mL of phosphate buffer saline (PBS) at 4°C. After gentle homogenisation, RBCs were washed
170 a first time by centrifuging the samples at 600*g* for 5 min to pellet the cells and discard the
171 supernatant. RBCs were then re-suspended in 1mL of ice-cold PBS, and one aliquot was frozen
172 at -80°C for later analysis of oxidative stress markers, while the second was stored at 4°C until
173 being used for mitochondrial measurements (see below). At this time, samples were washed a
174 second time as described above and re-suspended in 1mL of respiratory buffer MiR05 (0.5 mM
175 EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110
176 mM sucrose, free fatty acid bovine serum albumin (1 g/L), pH 7.1).

177

178 **Corticosterone (CORT) measurement**

179 Plasma total CORT levels were determined by immunoassay according to guidelines provided
180 by the manufacturer (Corticosterone EIA Kit, Enzo Life Sciences, USA) and as previously used in
181 this species^{46,47}. Intra-individual coefficient of variation based on duplicates was $9.86 \pm 2.98\%$.

182

183 **Oxidative stress measurements**

184 We evaluated a total of 14 markers of oxidative status (*i.e.* both oxidative damage and
185 antioxidant defences), 6 in plasma and 8 in RBCs (see Table 1 for a brief overview). Although
186 blood samples might not necessarily always reflect the whole-body response in terms of
187 oxidative stress, we combined two sample types providing different information (*i.e.* plasma
188 being an extra-cellular compartment integrating responses from different organs and RBC

189 being a specific cell type but possessing the full cellular machinery) to make the most of this
190 non-invasive and non-lethal sampling. Technical precision of measurements (CVs in %) was
191 evaluated based on 6 samples being analysed in duplicate (3 baseline and 3 T30 samples).

192

193 DNA oxidative damage

194 8-OHdG is one of the predominant forms of free radical-induced oxidative lesions on DNA. At
195 the cellular level, damaged guanine (8-OHdG) can be excised from genomic DNA by specific
196 repair enzymes, and enters the circulation before being eliminated through urine.
197 Consequently, plasma and urinary levels of this marker have the potential to reflect whole-
198 body oxidative stress status, and are both influenced by the level of damage and by the rate
199 of repair of such damage ¹².

200 The circulating concentration of 8-OHdG was quantified using a competitive
201 immunoassay (plasma diluted 1:5; DNA damage ELISA Kit, Enzo® Life Sciences, USA) as
202 previously described in king penguin ^{39,46}. Plasma DNA damage is expressed as ng of 8-
203 OHdG/mL, and intra-individual coefficient of variation based on duplicates was $12.44 \pm$
204 2.05%.

205 The 8-OHdG incorporated in RBC DNA was quantified using a competitive
206 immunoassay (300ng DNA, EpiQuick 8-OHdG DNA Damage Quantification Direct Kit
207 Colorimetric, Epigentek, USA). Briefly, DNA was extracted from 40µL of RBC-PBS solution
208 using a commercial spin-column method (NucleoSpin Blood QuickPure, Macherey-Nagel,
209 Germany) and quantified spectrophotometrically using a NanoDrop™ (ThermoFisher
210 Scientific™). Then, 300ng of purified DNA was used in the ELISA assay that was conducted
211 following manufacturer recommendations. RBC DNA damage is expressed as pg of 8-

212 OHdG/ μ g of DNA, and intra-individual coefficient of variation based on duplicates was $6.20 \pm$
213 1.19%.

214 Reactive Oxygen Metabolites (ROMs)

215 The concentration of reactive oxygen metabolites (ROMs) in the plasma was measured using
216 the d-ROM tests (5 μ L of plasma, Diacron International, Italy) following the manufacturer
217 instructions and as previously used in king penguin^{39,46}. The d-ROM test measures mostly
218 hydroperoxides (ROOH) as a marker of potential oxidative damage and has been validated
219 and extensively used in the past decade in birds⁴⁸. ROMs concentration is expressed as mg
220 of H₂O₂ equivalent/dL and intra-individual variation based on duplicates was $7.45 \pm 1.03\%$.

221

222 Protein carbonyl content

223 Carbonyl groups are introduced into the proteins by reactions with free radicals or lipid
224 peroxidation products, and damage produced by protein carbonylation is mostly irreversible
225¹². The carbonyl content of plasma and RBC samples (diluted to 1mg protein.mL⁻¹) was
226 quantified using a previously published protocol⁴⁹. First, nucleic acids were removed by
227 precipitation with streptomycin sulphonate (15 min at room temperature) and
228 centrifugation at 12,000g for 10min. Protein carbonyls were then derivatized to 2,4-
229 dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) for 1h at room
230 temperature. The pellet was precipitated with cold trichloroacetic acid at 20% and then
231 washed three times with a 1:1 solution of cold ethanol:ethyl acetate. The pellet was finally
232 re-suspended in 350 μ L of guanidine hydrochloride 6M. The absorbance of the samples was
233 read at 370nm and the mean absorbance of control tubes (incubated with 0.1M HCl instead
234 of DNPH) was then subtracted. We used the extinction coefficient of DNPH (0.022 μ mol.L⁻¹

235 $^1.\text{cm}^{-1}$) to calculate protein carbonyl content, which was expressed as nmol.mg^{-1} of protein.

236 Intra-individual variation based on duplicates was $14.58 \pm 2.27\%$.

237

238 Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) antioxidant activities

239 SOD is involved in the first step of the antioxidant enzymatic cascade catalysing the
240 dismutation of superoxide radical into oxygen and hydrogen peroxide. The enzymatic activity
241 of SOD in plasma (diluted 1:6) and RBC lysate (diluted 1:500) was measured with the SOD
242 activity kit (Enzo® Life Sciences, USA) following manufacturer instructions. This test
243 quantifies *in vitro* the kinetics of inhibition in superoxide formation resulting from SOD
244 antioxidant activity. SOD activity is expressed as U (units of enzymatic activity). mL^{-1} . Intra-
245 individual coefficient of variation based on duplicates was $12.00 \pm 1.96\%$.

246 Glutathione is used as a reductant by the GPx enzyme to scavenge deleterious
247 hydrogen peroxide. The enzymatic activity of GPx in plasma (diluted 1:10) and RBC lysate
248 (diluted 1:60) was measured with the RANSEL kit (Randox Laboratories, Crumlin, UK)
249 following manufacturer instructions. GPx activity is expressed as U.L^{-1} . Intra-individual
250 coefficient of variation based on duplicates was $9.09 \pm 1.40\%$.

251

252 Total and oxidized glutathione

253 Total glutathione (tGSH) content and oxidized glutathione (GSSG) content of RBC lysate
254 (diluted 1:480) were determined using DetectX® Glutathione fluorescent detection kit (Arbor
255 Assays, USA), following manufacturer instructions. Glutathione plays a key role in many
256 biological processes including the protection of cells against oxidation. We evaluated tGSH
257 content as an indicator of antioxidant protection, the amount of GSSG, and the ratio
258 GSSG/tGSH (which represent the proportion of oxidized glutathione) as indicators of the

259 oxidative challenge (*i.e.* the pro-oxidant power buffered by the glutathione system). Values
260 are respectively expressed as mmol.L^{-1} , and as a ratio of GSSG/tGSH (0 meaning that all
261 glutathione is reduced GSH, and 1 meaning that all glutathione is oxidized). Intra-individual
262 coefficient of variation based on duplicates was $1.41 \pm 0.41\%$.

263

264 Non-enzymatic (NE) antioxidant capacity OXY

265 We evaluated the NE antioxidant capacity of plasma (diluted 1:100) and red blood cell lysate
266 (diluted 1:2500) using the OXY-adsorbent test (Diacron International, Italy) following
267 manufacturer instructions. The OXY adsorbent test quantifies the ability of NE antioxidant
268 compounds to buffer a massive oxidation through hypochlorous acid (HClO). This assay
269 measures a variety of NE antioxidants, including vitamins, carotenoids, flavonoids and thiols.
270 NE antioxidant capacity is expressed as μM of HClO neutralized. L^{-1} for the plasma and as mM
271 of HClO neutralized. L^{-1} for RBCs. Intra-individual coefficient of variation based on duplicates
272 was $5.86 \pm 0.98\%$.

273

274 **Mitochondrial measurements in RBCs**

275 We followed the protocol we recently established for king penguins⁴³ to measure
276 mitochondrial function in fresh intact RBCs using a high-resolution respirometry system O2k
277 (Oroboros Instrument, Innsbruck, Austria) at the temperature of 38°C. We applied a serial
278 addition of chemicals to our RBC suspension in order to get a comprehensive assessment of
279 mitochondrial function. We used 2.5 μM of oligomycin to inhibit ATP synthesis, then 1 μM of
280 the mitochondrial uncoupler FCCP to stimulate respiration and finally 5 μM of Antimycin A to
281 fully inhibit mitochondrial respiration, as previously described⁴³. We computed four
282 parameters of mitochondrial respiration (*ROUTINE*, *OXPHOS*, *LEAK* and *ETS*) that were

283 normalized by the fresh mass of RBCs, and three different flux control ratios (FCRs) to better
284 characterize mitochondrial function of RBCs (see Table 1 and ⁴³ for details). Mitochondrial
285 respiration in intact cells is likely to be substrate and ADP limited, thereby not reflecting
286 maximal mitochondrial capacity. While our results in other bird species comparing intact and
287 permeabilized RBCs support this idea, they also suggest that mitochondrial parameters
288 measured in both conditions are highly correlated (Stier & Bize, pers. obs.). A summary of all
289 physiological markers is presented in Table 1 and original data is available at
290 [10.6084/m9.figshare.7042049](https://doi.org/10.6084/m9.figshare.7042049). All chemicals were purchased from Sigma-Aldrich (France)
291 unless specified otherwise.

292

293 **Statistics**

294 All statistical analyses were conducted using SPSS 20.0. We investigated the effects of
295 the acute restraint stress on corticosterone, mitochondrial parameters and oxidative stress
296 markers using repeated-measures Generalized Estimating Equations (GEE) with a Gaussian
297 distribution, with bird ID as the individual factor, sampling time (*i.e.* baseline vs. acute stress)
298 as the within-individual repeated effect, and sex as a fixed factor. We initially included the
299 interaction between sampling time and sex, but removed it from the final models since the
300 interaction was never significant (all $p > 0.07$). Standardized effect sizes d and their 95%
301 confidence interval for acute stress response were calculated following equations (4) and
302 (18)⁵⁰.

303 We investigated relationships between the magnitude of the relative CORT response (Δ
304 CORT) and the magnitude of both oxidative stress and mitochondrial responses to acute
305 restraint-stress (Δ oxidative stress and Δ mitochondria) in separate analyses. We used General
306 Linear Models (GLMs) with sex and Δ CORT as explanatory variables. Finally, we investigated

307 relationships between baseline CORT and respectively baseline mitochondrial parameters and
308 oxidative stress markers using GLMs with sex and baseline CORT as explanatory variables. In
309 both cases we calculated standardized effect sizes Z_r and their 95% confidence interval using
310 equations (11), (19) and (20)⁵⁰.

311 While measuring a broad range of physiological markers (*i.e.* 21 in our case, see Table 1)
312 brings detailed and valuable biological information, it comes at the cost of increasing the
313 number of statistical tests to perform, therefore increasing the likelihood of type I statistical
314 errors (*i.e.* false positives). Correcting for multiple testing comes at the cost of decreasing
315 considerably statistical power⁵¹, which in the case of experimental studies on wild animals like
316 king penguins could not be overcome by increasing substantially sample size for ethical and
317 practical constraints. We therefore chose to present results mostly as effect size reflecting the
318 magnitude of the biological effects we observe (Nakagawa et al. 2004), but still provide the
319 reader with information on p-values after false discovery rate (FDR) correction. We used the
320 two-stage sharpened method for FDR⁵².

321 Sample sizes vary slightly between markers due to 2 missing plasma samples and a few
322 failed laboratory assays (see full dataset available as ESM). Means are given \pm SE and p-values
323 ≤ 0.05 were considered as significant.

324

325 **Results**

326 **Corticosterone response to acute restraint-stress**

327 Plasma total CORT was on average 12.7 times higher in response to our standardized
328 restraint-stress protocol than at baseline levels (Wald $\chi^2 = 25.05$, $p < 0.001$, Fig. S1). We
329 found no significant effect of sex (Wald $\chi^2 = 2.54$, $p = 0.11$) on the CORT response.

330

331 **Oxidative stress response to acute restraint-stress**

332 Eight out of 14 oxidative stress markers were significantly affected by our standardized
333 restraint-stress protocol (Figs. 1, S2, S3 and Table S1).

334 *Oxidative damage* (Figs. 1 and S2): In the plasma, two markers of oxidative damage
335 (ROMs and protein carbonyl) decreased in response to acute restraint-stress, while 8-OHdG
336 increased. In RBCs, our two markers of oxidative damage were not significantly influenced by
337 acute restraint-stress, and while oxidized glutathione (GSSG) content increased in response
338 to acute stress, this was not the case for the proportion of glutathione oxidized
339 (GSSG/tGSH). Females had higher ROMs values (females = 6.81 ± 0.62 , males = 3.17 ± 0.38
340 mg of H₂O₂ equivalent/dL) but lower RBC 8-OHdG levels (females = 59.41 ± 3.31 , males = 73.49
341 ± 5.32 pg of 8-OHdG/ μ g of DNA) than males; other parameters did not significantly differ
342 between sexes (Table S1). The effect of acute restraint-stress on plasma protein carbonyl
343 was not significant anymore after FDR correction.

344 *Antioxidant defences* (Figs. 1 and S3): Plasma NE antioxidants decreased in response
345 to acute restraint-stress, while plasma SOD and GPx were not significantly impacted. In RBCs,
346 we found an increase in 3 markers of endogenous antioxidant defences (total glutathione,
347 SOD and GPx) in response to acute restraint-stress, while NE antioxidants were not
348 significantly affected (Fig. 2, Table S1). Markers of antioxidant defences were overall not
349 significantly different between males and females, except for plasma SOD with males having
350 higher levels than females (males = 16.31 ± 0.68 , females = 9.58 ± 0.49 U.mL⁻¹). The effects of
351 acute restraint-stress on plasma NE antioxidants and RBC GPx were not significant anymore
352 after FDR correction.

353

354 **Mitochondrial response to acute restraint-stress**

355 We found no significant effect of our standardized restraint-stress protocol (all $p > 0.22$, Figs.
356 2 and S3) or sex (all $p > 0.14$) on mitochondrial parameters (Table S1).

357

358 **Relationships between acute CORT response and acute changes in oxidative stress and** 359 **mitochondrial function in response to restraint-stress**

360 The magnitude of the CORT response (Δ CORT) was overall not predictive of the magnitude of
361 the changes in oxidative stress markers in response to acute restraint stress (Δ oxidative
362 stress), except for RBC NE antioxidants (Fig. 3 and Table S2). Birds having a marked CORT
363 response also had a positive change in RBC NE antioxidants in response to acute restraint-
364 stress, although this effect became non-significant after FDR correction.

365 The magnitude of the CORT response (Δ CORT) was overall not predictive of the magnitude of
366 the changes in mitochondrial function in response to acute restraint stress (Δ mitochondria),
367 except for *LEAK* respiration (Fig. 4 and Table S2). Birds having a marked CORT response
368 tended to have a positive change in *LEAK* respiration in response to acute restraint-stress,
369 although this effect became non-significant after FDR correction.

370

371 **Relationships between baseline CORT and baseline oxidative stress levels**

372 We found a positive relationship between baseline CORT and RBC total glutathione content,
373 and a marginally significant positive relationship between baseline CORT and RBC GPx
374 antioxidant activity (Fig. 5, Table S3). Baseline CORT was also negatively correlated with two
375 markers of oxidative damage, namely RBC proportion of oxidized glutathione and plasma
376 protein carbonyl. These effects did not remain significant after FDR corrections. All other
377 relationships between baseline CORT and baseline oxidative stress parameters were non-
378 significant (Fig. 5, Table S3).

379

380 **Relationship between baseline CORT and baseline mitochondrial parameters**

381 Baseline CORT was not significantly related to mitochondrial respiration rates (Fig.6, Table
382 S3). However, we found a marginally significant positive relationship between baseline CORT
383 and $FCR_{L/R}$ and a significant positive relationship between baseline CORT and $FCR_{L/ETS}$,
384 showing that individuals with higher baseline CORT had less efficient mitochondria (Fig.6,
385 Table S3). However, these effects did not remain significant after FDR corrections.

386

387 **Discussion**

388 We tested the hypothesis that king penguin, a colonial seabird species subject to
389 challenging environmental conditions and stressful stimuli while breeding on-land^{33,35,53},
390 could be naturally equipped to prevent stress-induced or GC-induced oxidative stress. We
391 investigated mitochondrial and oxidative stress responses to acute restraint-stress, and their
392 relationships with baseline (potentially mirroring exposure to chronic stress) and stress-
393 induced increase in corticosterone levels. In an attempt to achieve an integrative perspective
394 on individual mitochondrial and oxidative responses to stress, we measured both baseline
395 and stress-induced GC levels and investigated their relationship with an exhaustive number
396 of physiological markers (14 oxidative status markers and 7 markers of mitochondrial
397 functions). It follows that because of limited number of sampled birds (N = 24) – one of the
398 constraints of working on wild protected species – some of the effects we document became
399 non-significant after FDR correction. Yet, what is important to note is that FDR changes the
400 95% confidence intervals around estimates, not the estimates themselves. Thus, we
401 encourage readers to think about the meaning of effect sizes relative to one another, rather

402 than focusing solely on p -values⁵⁰. We nonetheless point out to effects that were no longer
403 significant after FDR in the following discussion.

404

405 Our results show an increase in endogenous antioxidant defences both in response to
406 acute restraint-stress and in relationship with increased baseline GC levels, whereas
407 oxidative damage levels were mostly reduced or not affected. We also found a reduction in a
408 proxy of mitochondrial efficiency to produce ATP (*i.e.* higher proportion of respiration being
409 linked to mitochondrial proton leak) both in relationship to the magnitude of the acute CORT
410 response and with increasing levels of baseline CORT. Because lower mitochondrial
411 efficiency is suggested to reduce ROS production⁵⁴, this suggests that an increase in acute
412 CORT response or in baseline CORT may be associated with a reduction in mitochondrial ROS
413 production in this species. Mitochondrial function was however not affected by acute
414 restraint-stress. King penguins seem therefore well equipped to buffer potential oxidative
415 damage arising from acute stress or high GC exposure, which contrasts to what has been
416 found in many other animals to date^{9,11,18,20-22}. Most of our knowledge so far on the
417 mechanisms linking acute/chronic stress or high GC exposure to oxidative stress comes from
418 laboratory animals adapted to unchallenging controlled environments. Therefore, our study
419 illustrates the importance of studying non-traditional model species in their natural habitat
420 to provide a comprehensive overview of the influence of stress exposure on both oxidative
421 stress and mitochondrial function, and to gain knowledge on how some species may thrive
422 in seemingly stressful environments.

423

424 **Acute stress: relationships between GCs, mitochondrial function and oxidative stress**

425 As expected, adult penguins experimentally restrained for a 30-minute-period experienced a
426 substantial increase in total CORT levels. However, this acute restraint had no significant
427 impact on mitochondrial function in RBCs at the population level, on average. Yet, birds with
428 the higher CORT response (Δ CORT) tended to be the ones exhibiting an increase in
429 mitochondrial proton leak in response to acute stress (Δ LEAK), which could contribute in
430 counter-balancing the potential pro-oxidant effects of high CORT during acute stress (see
431 below for details on mitochondrial uncoupling and ROS mitigation^{54,55}). Unfortunately, we
432 were not able to measure mitochondrial ROS production *per se*, and information in other
433 tissues than RBCs will also be needed to better understand the potential role of
434 mitochondria in the acute stress responses. Tissues can highly differ in their sensitivity to
435 GCs⁵⁶, so it is possible that acute increases in GCs may have more pronounced effects on
436 mitochondria from tissues being more directly involved in the “fight or flight” response, such
437 as skeletal muscles. Despite the absence of modifications in mitochondrial parameters after
438 30 minutes of restraint stress at the average population level, it is possible that effects could
439 become evident after a more prolonged restraint stress, or even during/following the
440 recovery from this acute stress.

441 Oxidative damage levels changed markedly in response to acute restraint-stress (4
442 markers out of 7). Surprisingly, two markers of oxidative damage decreased in response to
443 acute stress. These findings contrast with the idea that acute stress may increase oxidative
444 damage²⁰⁻²², and might be explained by the concurrent increase in several antioxidant
445 mechanisms (3 markers out of 7; see below). Nevertheless, we found an increase in plasma
446 8-OHdG, but not in RBC 8-OHdG, in response to acute stress. We have to keep in mind that
447 plasma 8-OHdG integrates whole-body DNA damage, but is also influenced by repair levels.
448 Therefore, two non-mutually exclusive hypotheses could explain these results. First,

449 elevated plasma 8-OHdG levels may indicate increased repair of DNA damage at the cellular
450 level to keep 8-OHdG integrated within genomic DNA at a constant level, explaining the lack
451 of increase in RBC 8-OHdG. Second, DNA damage levels in response to acute stress may
452 differ between RBCs and other tissues. Hence, an increase in plasma 8-OHdG levels may
453 reflect increased oxidative stress in other tissues than RBCs, potentially in some tissues being
454 more sensitive to acute stress (*e.g.* skeletal muscle). Finally, the elevated levels of oxidized
455 glutathione we found in response to acute stress could be indicative of an increased amount
456 of ROS being quenched by the glutathione system. Whether these ROS could originate from
457 the mitochondria or from other sources (*e.g.* autoxidation of haemoglobin or activity of
458 enzymes such as xanthine oxidase) remains nonetheless unknown, calling for direct
459 measurement of both mitochondrial and non-mitochondrial ROS production during acute
460 stress in the future.

461 Antioxidant defences were also affected by acute stress (4 markers out of 7). The
462 only marker that decreased in response to acute stress was plasma non-enzymatic
463 antioxidant (sometimes referred to as TAC, total antioxidant capacity), which is in
464 accordance with previous studies in birds ^{19-21,24}. This antioxidant marker reflects mostly
465 antioxidant compounds acquired through the diet ⁵⁷, and the observed decrease in plasma
466 TAC probably reflected their consumption to buffer increase in ROS production resulting
467 from acute stress. In contrast, several RBC antioxidant systems (glutathione, GPx and SOD)
468 increased, suggesting an activation of cellular endogenous antioxidant defences in response
469 to acute stress. This activation of endogenous antioxidants might have been greater than the
470 potential increase in ROS, thereby explaining why both plasma ROMs and protein carbonyl
471 levels decreased in response to acute restraint-stress.

472 We found no significant relationship between the intensity of the acute CORT
473 response and the magnitude of the oxidative stress response (Fig. 3) except for RBC NE
474 antioxidants, suggesting that these two processes are potentially independently regulated.
475 Yet, experiments inducing an acute increase in exogenous GCs (without inducing restraint-
476 stress) and measuring oxidative stress levels in response to this challenge are needed to
477 exclude a direct role of GCs. Beside GCs, other hormones involved in the stress response
478 could explain variations in oxidative stress levels resulting from acute restraint-stress. For
479 instance, adrenaline increases very rapidly during the first seconds to minutes after stress
480 exposure, and has been shown to affect oxidative balance *in vitro*⁵⁸.

481 Although restraint stress is a widely used method to measure the ability to mount an
482 acute stress response in free-living animals, this stressor is not directly comparable to
483 natural stressors (*e.g.* predator encounter). Future studies thus need to extend our
484 evaluation of the oxidative stress response to more natural acute stressors (*e.g.* simulated
485 predation event). This should enable a clear evaluation of the relevance of using restraint-
486 stress as a methodological approach to study oxidative stress response in wild animals.

487

488 **Relationships between baseline GCs, mitochondrial function and oxidative stress**

489 Although relationships between baseline CORT levels and mitochondrial function were only
490 moderate (and non-significant after FDR correction), our results suggest that individuals with
491 higher baseline CORT had slightly less efficient mitochondria at producing ATP for a given
492 amount of O₂ and substrate consumed (*i.e.* higher relative proton leak). This result might be
493 surprising at a first glance, since one would expect individuals to optimize mitochondrial
494 efficiency under harsh/demanding environmental conditions, which has been for instance
495 demonstrated in king penguin chicks during their wintering fast⁵⁹. Yet, decreasing

496 mitochondrial efficiency is one known mechanism limiting ROS production, and thus
497 oxidative stress^{54,55}, even if the role of this mechanism is less established in birds than in
498 other animals^{60,61}. Decreasing mitochondrial efficiency in response to increased baseline
499 CORT levels could be one useful mechanism to mitigate oxidative stress during chronic
500 exposure to stressful stimuli.

501 Higher baseline CORT levels were not associated with higher oxidative damage levels,
502 confirming our previous findings in this species³⁹. Moreover, high CORT levels were
503 associated to a low proportion of oxidized glutathione and low levels of protein oxidative
504 damage, suggesting a potential beneficial effect of baseline CORT to maintain low levels of
505 oxidative damage, although these effects were moderate (and non-significant after FDR
506 correction). Besides changes in mitochondrial efficiency (see above), low levels of oxidative
507 damage could be explained by an increase in antioxidant defences in response to elevated
508 baseline CORT levels. Indeed, baseline CORT was positively correlated to the activity of the
509 glutathione antioxidant system (*i.e.* total glutathione and GPx activity), suggesting that high
510 baseline CORT could potentially trigger this pathway in our study species. The correlative
511 nature of these results and the limitations inherent to the use of baseline CORT as an
512 indicator of chronic stress prevent us however from reaching firm conclusions here. Indeed,
513 baseline CORT could potentially reflect stress responsiveness rather than being a proxy of
514 chronic stress exposure (*i.e.* as suggested by a positive correlation between baseline and T30
515 levels in our current dataset, Fig. S1), and more recently CORT has been suggested to reflect
516 more metabolic demand than 'stress'⁶². Future experimental studies should test if chronic
517 stress and/or increased GC levels trigger an increase in endogenous antioxidants defences
518 and a decrease in mitochondrial efficiency to prevent oxidative stress.

519

520 **Conclusion**

521 In contrast to most previous studies^{9,18,20-22}, our results show mostly no change or
522 even a decrease in oxidative damage markers in relation to acute stress and high baseline or
523 stress-induced GC levels. These findings are consistent with the hypothesis that animals
524 dealing with repeated stressors in their environment, such as king penguins, may possess
525 physiological mechanisms preventing oxidative damage to occur as a consequence of stress
526 exposure. Interestingly, similar findings in captive house sparrows linking acute restraint-
527 stress¹⁹ and experimental chronic elevation of GC hormones (*i.e.* CORT implant)⁶³ to
528 reduced oxidative damage have been recently documented. In penguins, part of this
529 resistance to oxidative stress might be related to physiological adaptations to marine life and
530 long-term fasting as recently shown in this species⁶⁴. The role of mitochondrial uncoupling
531 (*i.e.* decreased efficiency) as a potential mechanism of oxidative stress prevention during
532 chronic stress exposure deserves further investigation, especially in the light of a recent
533 study showing a reduced ROS production in response to CORT treatment despite no effect
534 on mitochondrial bioenergetics in captive lizards⁶⁵. Experimental manipulations of GC levels
535 (*e.g.* via exogenous administration) and chronic stress (*e.g.* repeated simulated predation
536 events) are now required to demonstrate causal effects of GCs and chronic stress on
537 mitochondrial function and oxidative stress in wild animals.

538

539 **Competing interests**

540 We declare having no competing interests

541

542 **Data availability**

543 The dataset used in this manuscript is available at: <https://doi.org/10.6084/m9.figshare.7042049.v1>

544 **Author's contribution**

545 AS designed the study, conducted fieldwork and laboratory analyses, analysed the data and
546 wrote the paper. QS, EL, JPR contributed to data collection in the field, and commented on
547 the manuscript. MH, DR and PB contributed to the original idea, data analyses and
548 commented on the manuscript. VAV contributed to data analyses and writing the paper.

549

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557

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716

Table 1: Summary of physiological parameters measured in this study and their biological meaning

Physiological parameters	Biological meaning
Glucocorticoids <i>Baseline CORT</i> <i>CORT T30</i> <i>ΔCORT</i>	Baseline corticosterone levels Corticosterone absolute stress response to 30 min of standardized handling Corticosterone relative stress response to 30 min of handling (T30 minus baseline)
Oxidative damage <i>Plasma ROMs</i> <i>Plasma protein carbonyl</i> <i>Plasma 8-OHdG</i> <i>RBC protein carbonyl</i> <i>RBC 8-OHdG</i> <i>RBC GSSG</i> <i>RBC GSSG/tGSH</i>	Reactive Oxygen Metabolites in the plasma, a marker of early oxidative damage Oxidative damage to proteins in the plasma (<i>i.e.</i> extra-cellular) Circulating levels of 8-OHdG, a product of oxidative damage to DNA reflecting whole-body oxidative stress and being influenced by rates of both damage and repair Oxidative damage to proteins in the red blood cells (<i>i.e.</i> intra-cellular) Oxidative damage to DNA being integrated into cellular DNA of red blood cells Glutathione in red blood cells that has been oxidized by reactive oxygen species Proportion of glutathione in red blood cells being oxidized relative to the overall quantity of glutathione
Antioxidant defences <i>RBC tGSH</i> <i>RBC GPx</i> <i>RBC SOD</i> <i>RBC OXY</i> <i>Plasma GPx</i> <i>Plasma SOD</i> <i>Plasma OXY</i>	Red blood cell total (reduced + oxidized) content of the endogenous antioxidant glutathione Activity of the endogenous antioxidant enzyme glutathione peroxidase in red blood cell Activity of the endogenous antioxidant enzyme superoxide dismutase in red blood cell Total content of non-enzymatic antioxidants in red blood cells Activity of the endogenous antioxidant enzyme glutathione peroxidase in plasma Activity of the endogenous antioxidant enzyme superoxide dismutase in plasma Total content of non-enzymatic antioxidants in the plasma (mostly acquired through the diet)
Mitochondrial respiration rates of intact RBCs <i>ROUTINE</i> <i>OXPHOS</i> <i>LEAK</i> <i>ETS</i>	Mitochondrial respiration of intact red blood cells with their endogenous substrates and ADP Mitochondrial respiration linked to ATP synthesis Mitochondrial respiration linked to mitochondrial proton leak Maximal mitochondrial respiration of intact red blood cells with their endogenous substrates
Mitochondrial Flux Control Ratios of intact RBCs <i>FCR_{L/R}</i> <i>FCR_{L/ETS}</i> <i>FCR_{R/ETS}</i>	Proportion of mitochondrial ROUTINE respiration being linked to proton leak, indicating mitochondrial efficiency under endogenous cellular conditions Proportion of mitochondrial maximal respiration being linked to proton leak, indicating mitochondrial efficiency under stimulated cellular conditions Proportion of mitochondrial maximal respiration being used under endogenous cellular conditions, indicating the activation state of the mitochondria

717

717 **Figure captions:**

718

719 **Fig. 1: Effects of acute restraint-stress on oxidative stress parameters.** Standardized effect sizes d
720 and their 95% confidence intervals are shown. Statistically significant effects (95% CI not overlapping
721 0) are presented in black, while non-significant ones (95% CI overlapping 0) are presented in light
722 grey. Details on statistical tests are provided in Table S1. Parameters remaining significant after false
723 discovery rate (FDR) correction are marked with a *.

724

725 **Fig. 2: Effects of acute restraint-stress on mitochondrial parameters.** Standardized effect size d
726 and their 95% confidence interval are shown. Non-significant effects are presented in light grey.
727 Details on statistical tests are provided in Table S1.

728

729 **Fig. 3: Relationships between corticosterone (CORT) and oxidative stress responses to an acute**
730 **restraint-stress.** Standardized effect size Zr and their 95% confidence interval are shown. Statistically
731 significant effects are presented in black, and non-significant ones in light grey. Details on statistical
732 tests are provided in Table S2. Parameters remaining significant after false discovery rate (FDR)
733 correction are marked with a *.

734

735 **Fig. 4: Relationships between corticosterone (CORT) and mitochondrial stress responses to an**
736 **acute restraint-stress.** Standardized effect size Zr and their 95% confidence interval are shown.
737 Statistically significant effects are presented in black, marginally-significant ones ($p \leq 0.10$) in dark
738 grey with open symbol, and non-significant ones in light grey. Details on statistical tests are provided
739 in Table S2. Parameters remaining significant after false discovery rate (FDR) correction are marked
740 with a *.

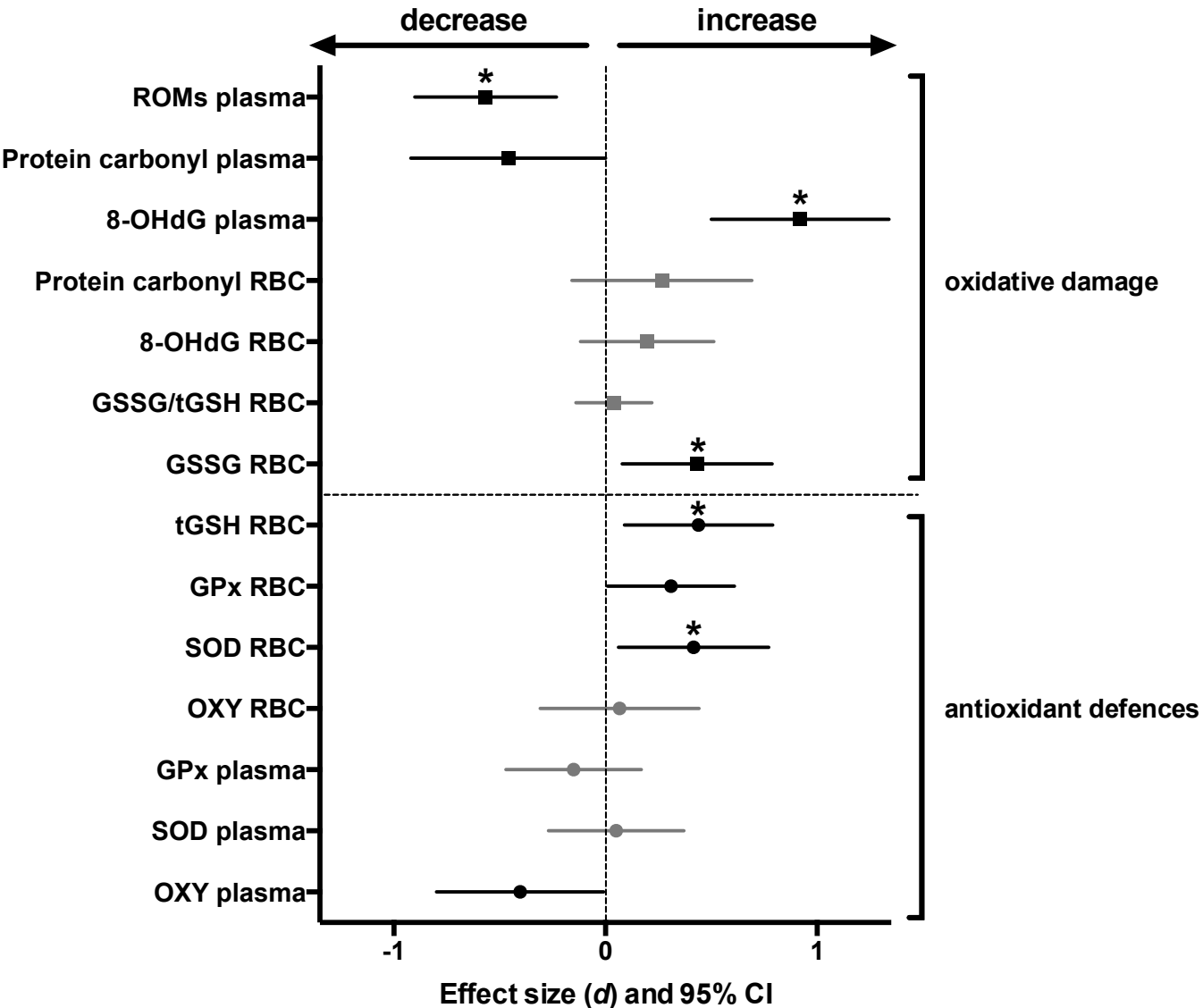
741

742 **Fig. 5: Relationships between baseline levels of corticosterone (CORT) and oxidative stress**
743 **markers.** Standardized effect size Zr and their 95% confidence interval are shown. Statistically
744 significant effects are presented in black, marginally-significant ones ($p \leq 0.10$) in dark grey with open
745 symbol, and non-significant ones in light grey. Details on statistical tests are provided in Table S3.
746 Parameters remaining significant after false discovery rate (FDR) correction are marked with a *.

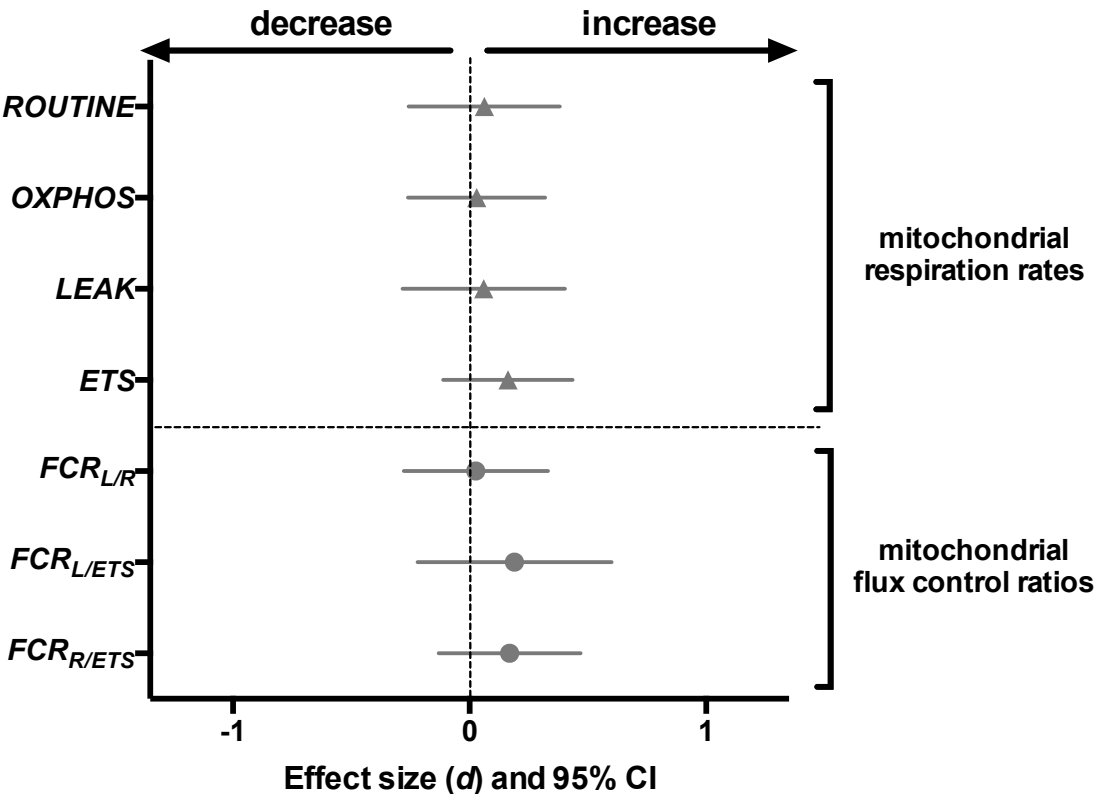
747

748 **Fig. 6: Relationships between baseline levels of corticosterone (CORT) and mitochondrial**
749 **parameters.** Standardized effect size Zr and their 95% confidence interval are shown. Statistically
750 significant effects are presented in black, marginally-significant ones ($p \leq 0.10$) in dark grey with open
751 symbol, and non-significant ones in light grey. Details on statistical tests are provided in Table S3.
752 Parameters remaining significant after false discovery rate (FDR) correction are marked with a *.

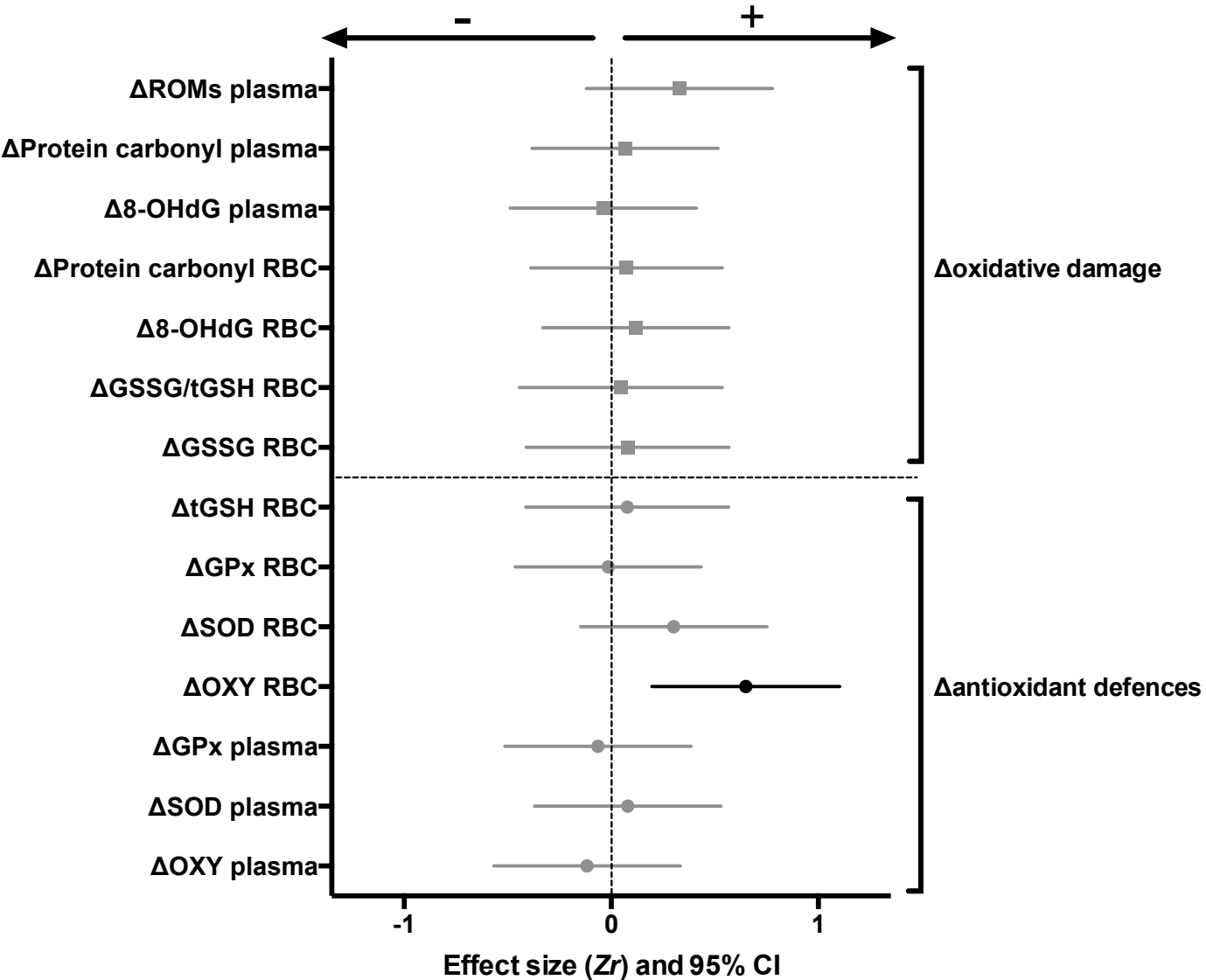
Response to acute restraint-stress



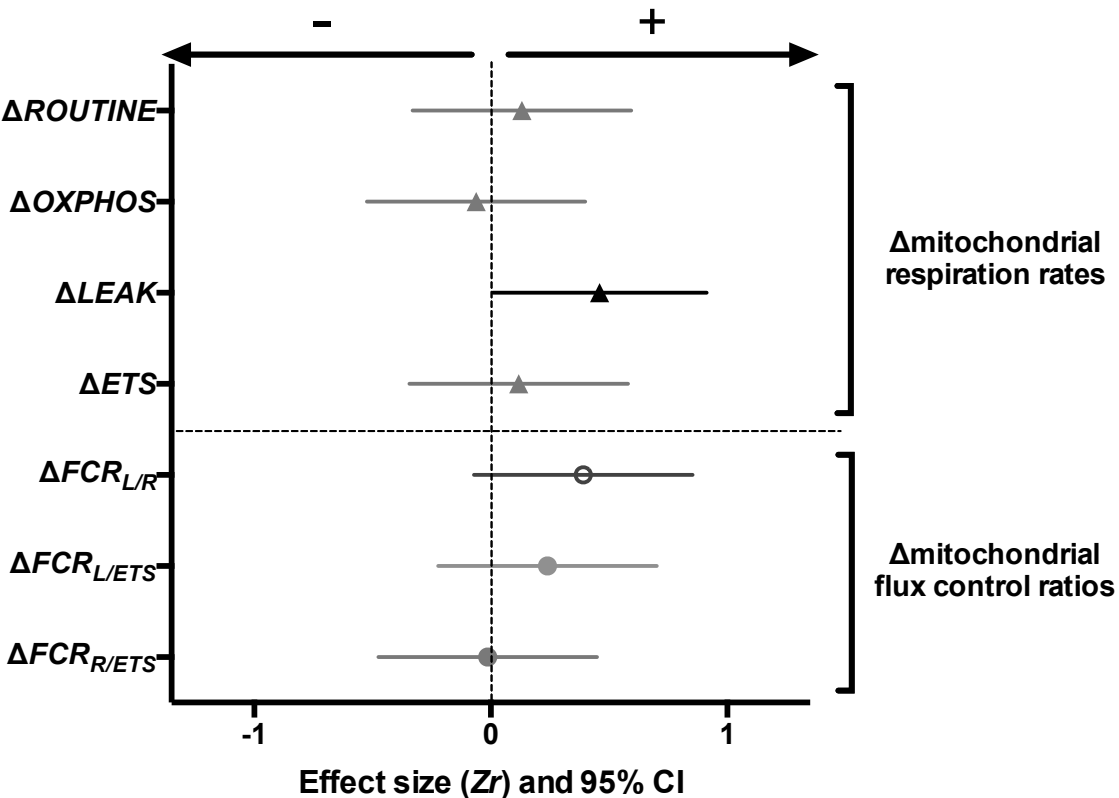
Response to acute restraint-stress



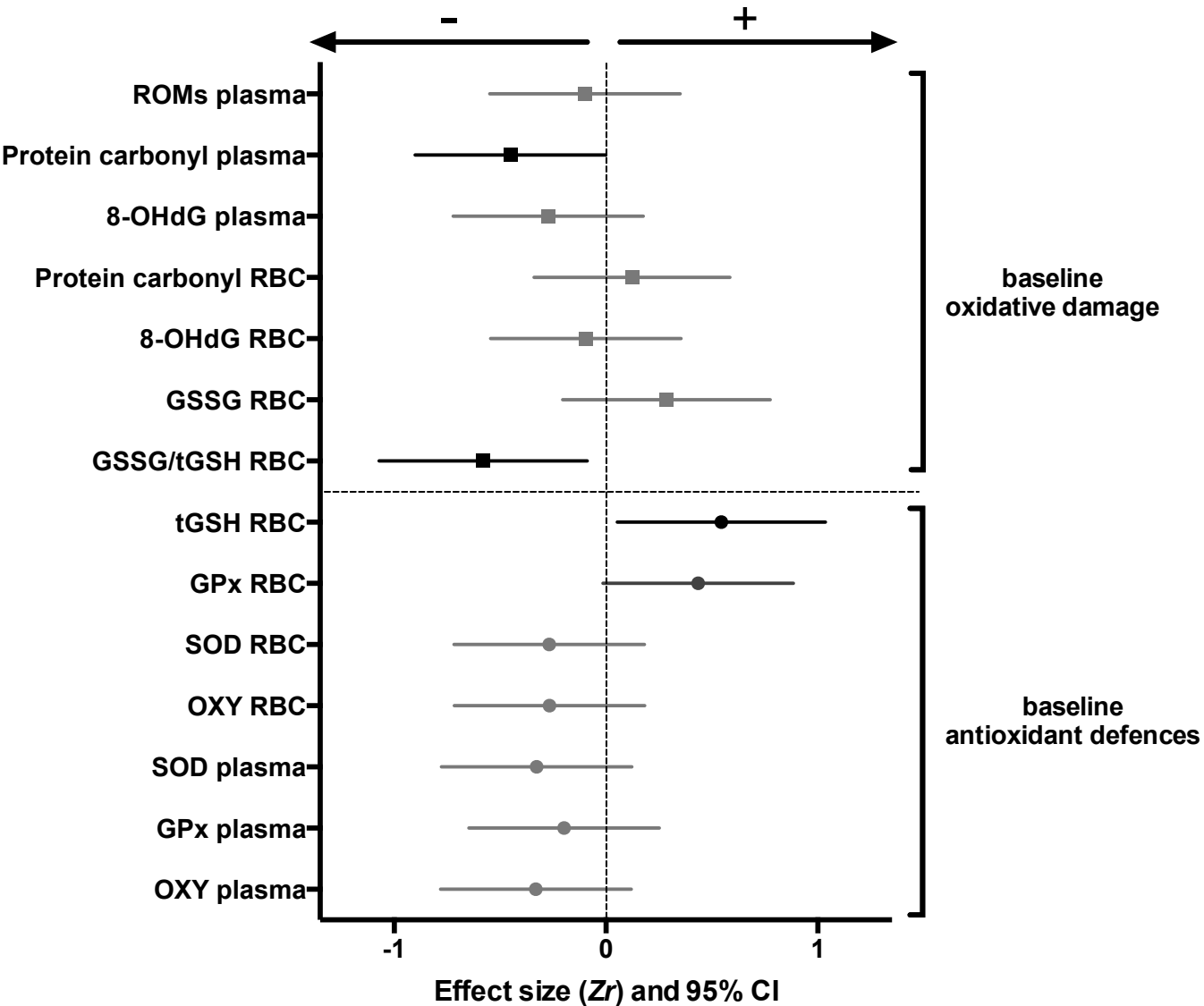
Relationship with acute CORT response (Δ CORT)



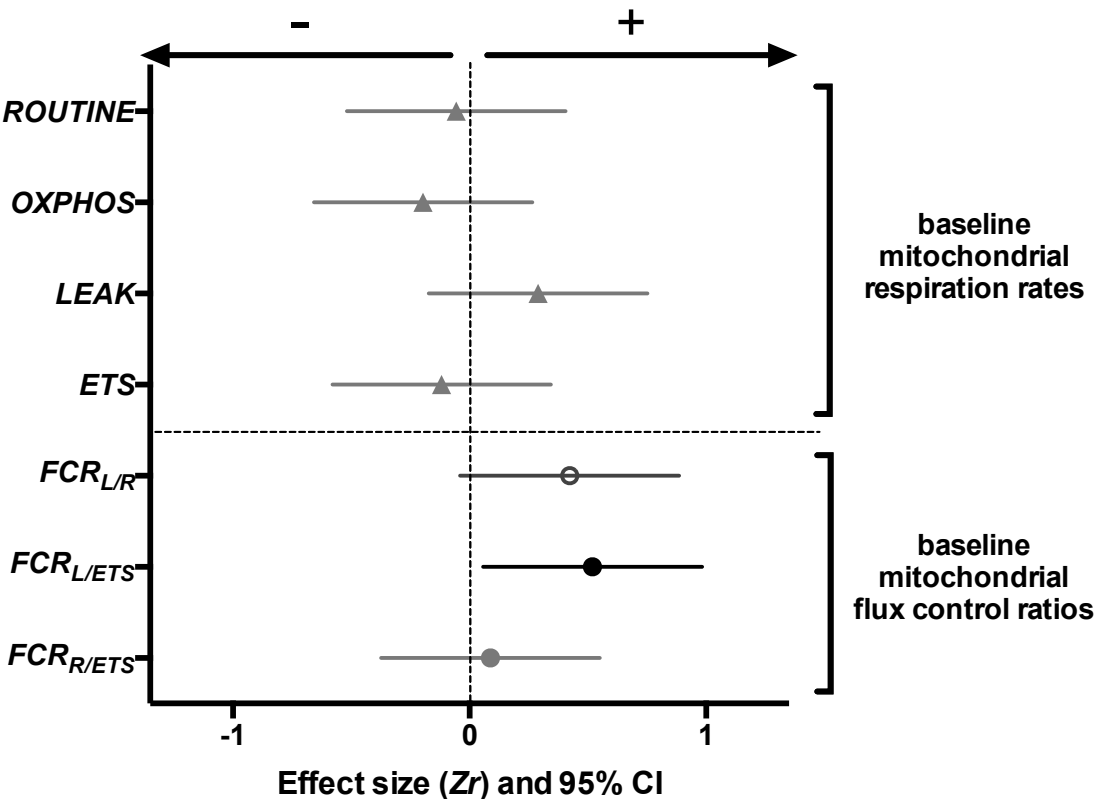
Relationship with acute CORT response (Δ CORT)



Relationship with baseline CORT levels



Relationship with baseline CORT levels



Electronic Supplementary Material (ESM)

Oxidative stress and mitochondrial responses to stress exposure suggest that king penguins are naturally equipped to resist stress

Antoine Stier, Quentin Schull, Pierre Bize, Emilie Lefol, Mark Haussmann, Damien Roussel, Jean-Patrice Robin, & Vincent A Viblanc

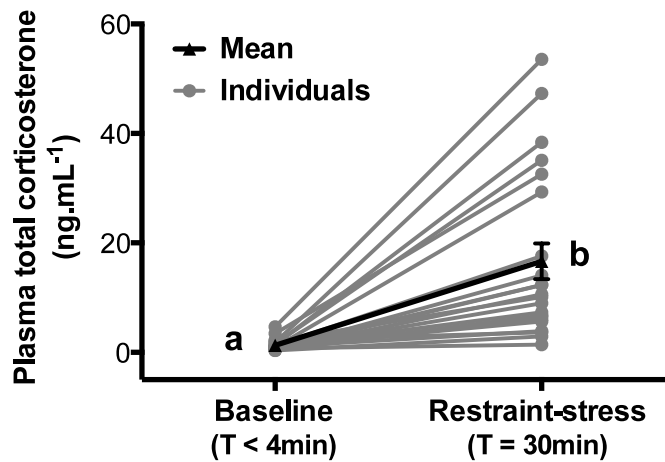


Fig. S1: Plasma concentration of total corticosterone at baseline (< 4min of capture) and in response to an acute restraint-stress protocol (*i.e.* 30 min of standardized handling). Individual responses are shown in grey and the mean population response \pm SE is shown in black. Different letters indicate significant differences between the two sampling times (*i.e.* populations means) according to statistical model presented in the main text.

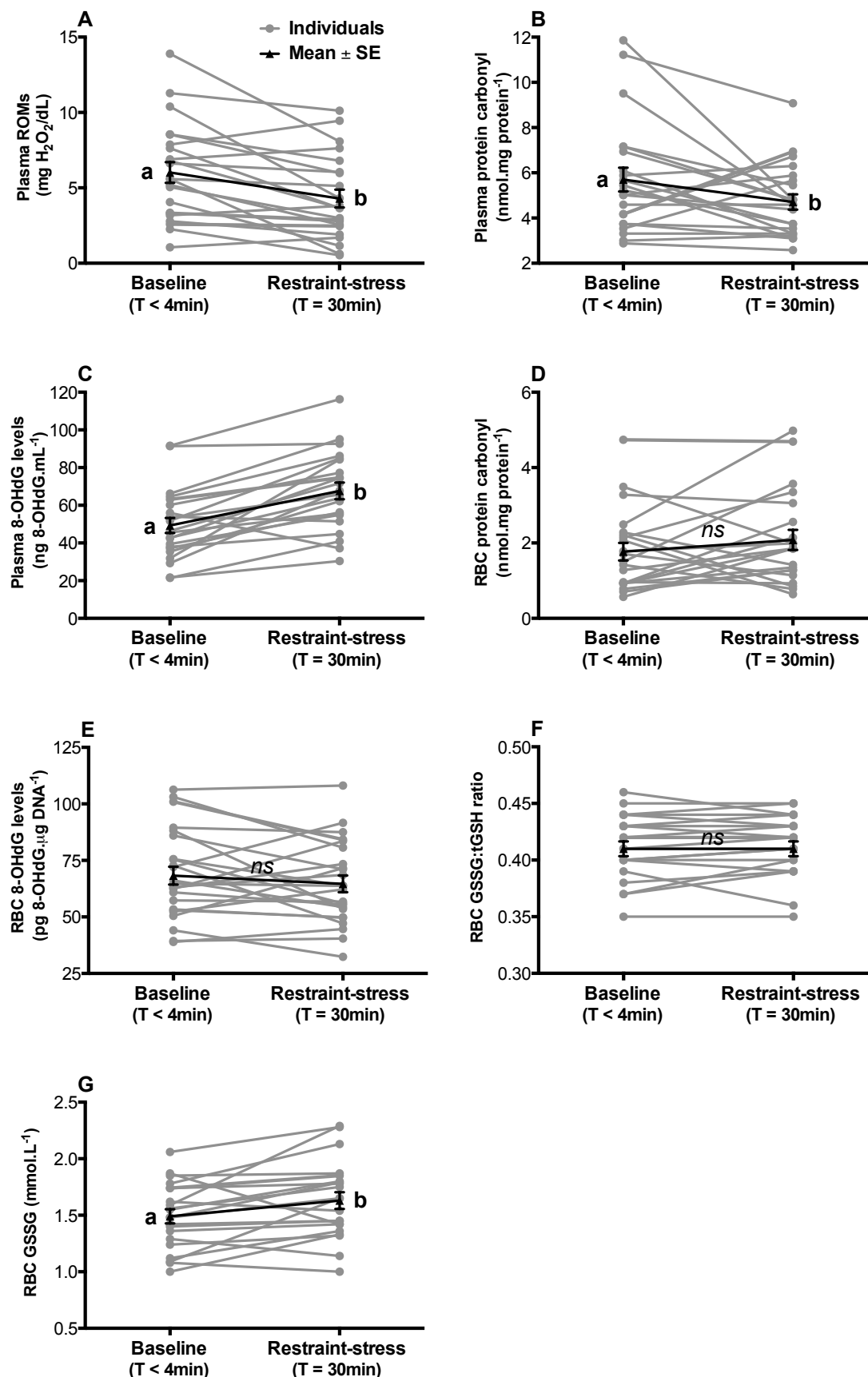


Fig. S2: Oxidative damage markers at baseline (< 4min of capture) and in response to an acute restraint-stress protocol (*i.e.* 30 min of standardized handling). (A) Plasma ROMs, (B) Plasma protein carbonyl, (C) Plasma 8-OHdG, (D) RBC protein carbonyl, (E) RBC 8-OHdG, (F) RBC GSSG:tGSH ratio and (G) RBC GSSG. The biological meaning of the different markers is explained in Table 1. Individual responses are shown in grey and the mean population response ± SE is shown in black. Different letters indicate significant differences between the two sampling times (*i.e.* populations means) according to statistical models presented Table S1.

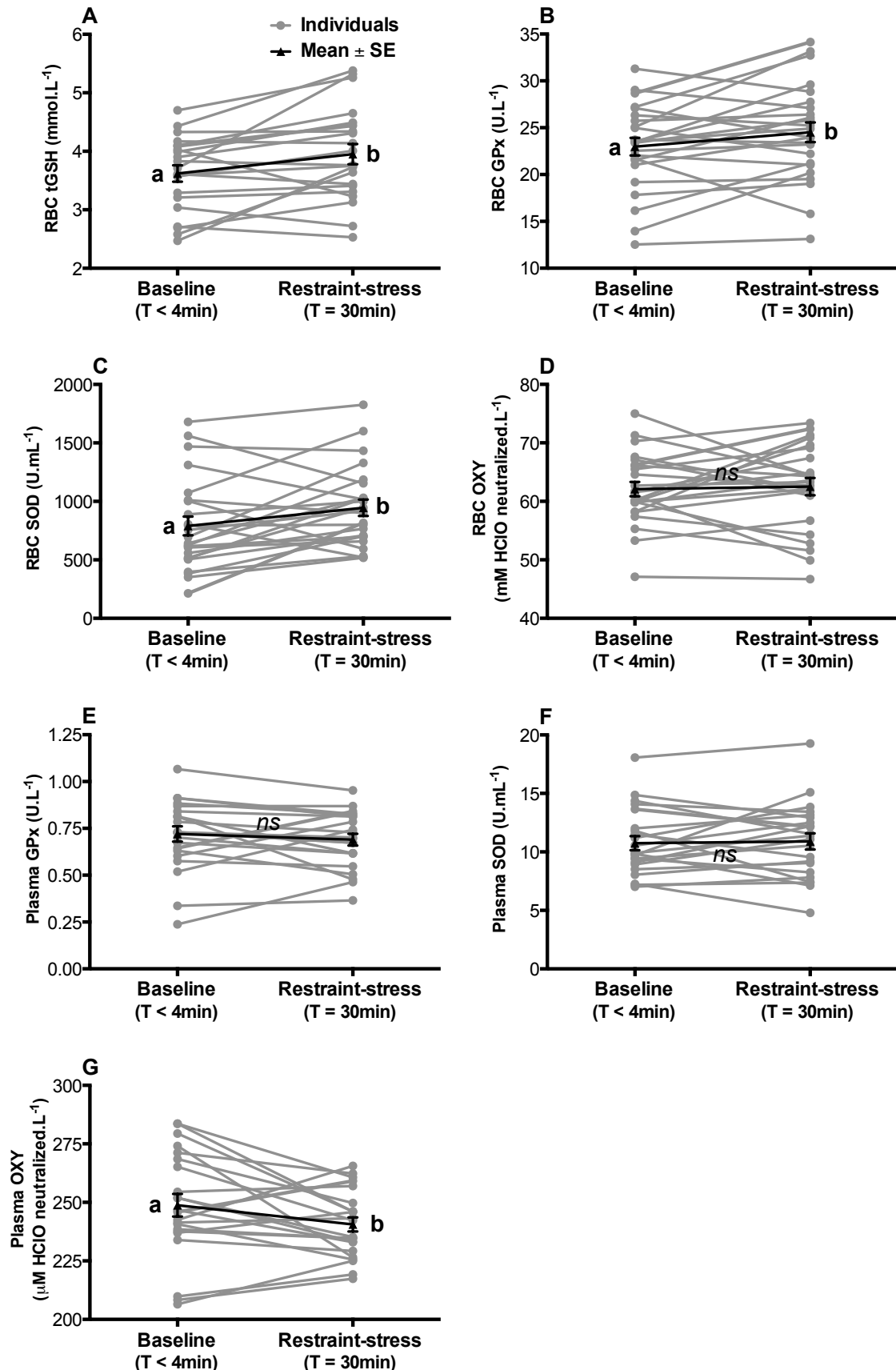


Fig. S3: Antioxidant markers at baseline (< 4min of capture) and in response to an acute restraint-stress protocol (*i.e.* 30 min of standardized handling). (A) RBC tGSH, (B) RBC GPx, (C) RBC SOD, (D) RBC OXY, (E) Plasma GPx, (F) Plasma SOD and (G) Plasma OXY. The biological meaning of the different markers is explained in Table 1. Individual responses are shown in grey and the mean population response \pm SE is shown in black. Different letters indicate significant differences between the two sampling times (*i.e.* populations means) according to statistical models presented Table S1.

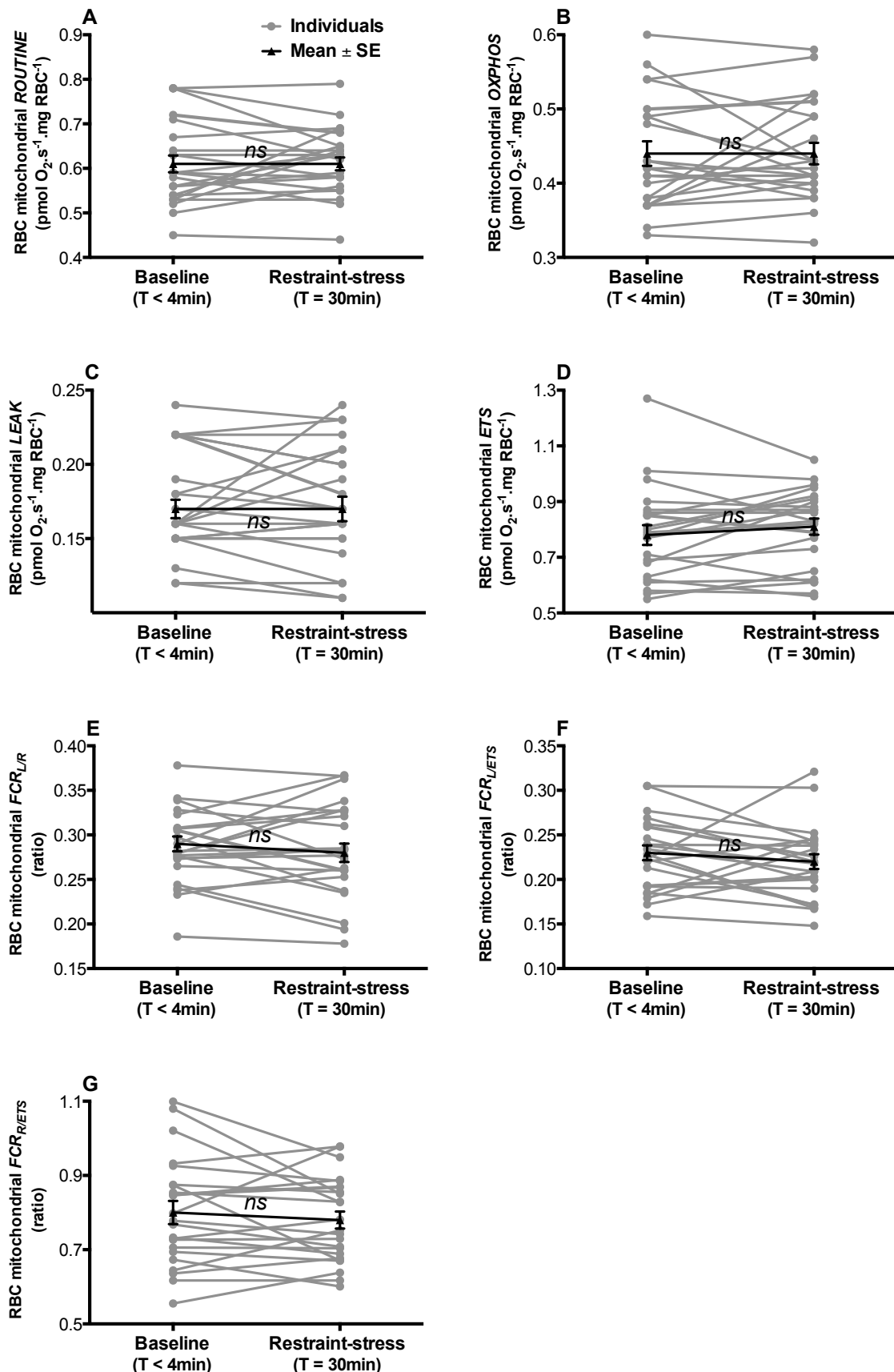


Fig. S4: Mitochondrial respiration rates and flux control ratios at baseline (< 4min of capture) and in response to an acute restraint-stress protocol (*i.e.* 30 min of standardized handling). (A) ROUTINE, (B) OXPPOS, (C) LEAK, (D) ETS, (E) FCR_{L/R}, (F) FCR_{L/ETS} and (G) FCR_{R/ETS}. The biological meaning of the different markers is explained in Table 1. Individual responses are shown in grey and the mean population response \pm SE is shown in black. Different letters indicate significant differences between the two sampling times (*i.e.* populations means) according to statistical models presented Table S1.

Table S1: Summary of the statistical models (GEEs) testing the effects of acute restraint-stress exposure and sex on: (A) oxidative stress parameters and (B) mitochondrial parameters. Significant parameters ($p \leq 0.05$) are reported in bold. Parameters remaining significant after false discovery rate (FDR) correction are underlined and marked with a *. Estimates are given for T30 samples and for females.

	Acute restraint-stress		Sex	
A) Oxidative stress markers	Estimate \pm SE	p-value (χ^2)	Estimate \pm SE	p-value (χ^2)
<u>Plasma ROMs</u>	-1.73 \pm 0.44	<u>< 0.001 (15.48)*</u>	3.64 \pm 0.83	<u>< 0.001 (19.07)*</u>
Plasma protein carbonyl	-0.98 \pm 0.48	0.039 (4.27)	0.27 \pm 0.69	0.69 (0.15)
<u>Plasma 8-OHdG</u>	18.40 \pm 3.13	<u>< 0.001 (34.50)*</u>	7.62 \pm 7.74	0.33 (0.97)
RBC protein carbonyl	0.31 \pm 0.24	0.20 (1.65)	-0.02 \pm 0.46	0.97 (0.00)
RBC 8-OHdG	-3.69 \pm 2.93	0.21 (1.59)	-14.08 \pm 6.27	0.025 (5.04)
<u>RBC GSSG</u>	0.14 \pm 0.05	<u>0.008 (7.09)*</u>	0.02 \pm 0.12	0.86 (0.03)
RBC GSSG/GSH	0.001 \pm 0.003	0.68 (0.17)	0.010 \pm 0.011	0.39 (0.75)
<u>RBC total GSH</u>	0.33 \pm 0.12	<u>0.006 (7.62)*</u>	-0.07 \pm 0.29	0.82 (0.05)
RBC GPx	1.53 \pm 0.71	0.031 (4.63)	-0.01 \pm 1.82	0.99 (0.00)
<u>RBC SOD</u>	154.6 \pm 61.8	<u>0.012 (6.26)*</u>	-0.1 \pm 134.0	0.99 (0.00)
RBC OXY	0.44 \pm 1.28	0.73 (0.12)	-3.48 \pm 2.25	0.12 (2.39)
Plasma GPx	-0.026 \pm 0.028	0.35 (0.88)	0.097 \pm 0.065	0.14 (2.19)
Plasma SOD	0.15 \pm 0.49	0.76 (0.09)	-2.73 \pm 1.05	0.009 (6.80)
Plasma OXY	-8.23 \pm 3.85	0.033 (4.57)	3.40 \pm 6.72	0.61 (0.26)
B) Mitochondrial parameters	Estimate \pm SE	p-value (χ^2)	Estimate \pm SE	p-value (χ^2)
ROUTINE	0.005 \pm 0.014	0.70 (0.15)	0.027 \pm 0.032	0.40 (0.71)
LEAK	0.001 \pm 0.053	0.84 (0.04)	0.019 \pm 0.013	0.15 (2.09)
OXPHOS	0.004 \pm 0.012	0.72 (0.12)	0.007 \pm 0.027	0.79 (0.07)
ETS	0.026 \pm 0.021	0.23 (1.45)	0.039 \pm 0.058	0.51 (0.43)
FCR _{L/R}	-0.001 \pm 0.008	0.87 (0.03)	0.020 \pm 0.018	0.26 (1.25)
FCR _{L/ETS}	-0.008 \pm 0.008	0.36 (0.85)	0.018 \pm 0.014	0.22 (1.54)
FCR _{R/ETS}	-0.023 \pm 0.020	0.26 (1.29)	0.008 \pm 0.049	0.87 (0.03)

Table S2: Summary of the statistical models (GEEs) testing the relationships between the magnitude of the relative corticosterone acute stress response (Δ CORT) and the magnitude of the: (A) acute oxidative stress responses (Δ oxidative stress), and (B) acute mitochondrial responses (Δ mitochondria). Significant parameters ($p \leq 0.05$) are reported in bold, and parameters being marginally significant ($p \leq 0.10$) are reported in italic. Parameters remaining significant after false discovery rate (FDR) correction are underlined and marked with a *. Estimates for sex are given for females.

	Δ CORT		Sex	
	Estimate \pm SE	p-value (F)	Estimate \pm SE	p-value (F)
A) Δ Oxidative stress markers				
Δ Plasma ROMs	0.049 \pm 0.033	0.15 (2.26)	1.00 \pm 0.95	0.30 (1.12)
Δ Plasma protein carbonyl	0.011 \pm 0.038	0.77 (0.09)	-0.21 \pm 1.09	0.85 (0.04)
Δ Plasma 8-OHdG	-0.041 \pm 0.245	0.87 (0.03)	4.30 \pm 7.07	0.55 (0.37)
Δ RBC protein carbonyl	0.006 \pm 0.018	0.76 (0.10)	-0.331 \pm 0.55	0.55 (0.36)
Δ RBC 8-OHdG	0.12 \pm 0.24	0.61 (0.27)	9.88 \pm 6.92	0.17 (2.04)
Δ RBC GSSG	0.002 \pm 0.005	0.75 (0.10)	-0.03 \pm 0.13	0.84 (0.04)
Δ RBC GSSG/GSH	0.000 \pm 0.000	0.86 (0.035)	-0.008 \pm 0.006	0.21 (1.75)
<hr/>				
Δ RBC total GSH	0.003 \pm 0.011	0.76 (0.10)	-0.02 \pm 0.30	0.96 (0.00)
Δ RBC GPx	-0.004 \pm 0.058	0.95 (0.10)	-1.37 \pm 1.69	0.43 (0.66)
Δ RBC SOD	6.81 \pm 4.99	0.19 (1.87)	-39.94 \pm 144.13	0.79 (0.08)
ΔRBC OXY	0.29 \pm 0.08	0.003 (11.6)	0.81 \pm 2.45	0.74 (0.11)
Δ Plasma GPx	-0.001 \pm 0.002	0.78 (0.08)	-0.049 \pm 0.063	0.45 (0.60)
Δ Plasma SOD	0.013 \pm 0.037	0.73 (0.12)	-1.00 \pm 1.07	0.37 (0.86)
Δ Plasma OXY	-0.14 \pm 0.28	0.62 (0.26)	-14.03 \pm 8.20	0.10 (2.93)
B) Δ Mitochondrial parameters				
Δ ROUTINE	0.001 \pm 0.033	0.58 (0.32)	-0.014 \pm 0.032	0.67 (0.19)
ΔLEAK	0.001 \pm 0.000	0.050 (4.40)	0.009 \pm 0.012	0.48 (0.52)
Δ OXPPOS	0.000 \pm 0.000	0.80 (0.07)	-0.023 \pm 0.028	0.42 (0.68)
Δ ETS	0.001 \pm 0.002	0.62 (0.26)	0.031 \pm 0.049	0.53 (0.41)
<hr/>				
Δ FCR _{L/R}	<i>0.001 \pm 0.001</i>	<i>0.09 (3.12)</i>	0.019 \pm 0.017	0.28 (1.23)
Δ FCR _{L/ETS}	0.001 \pm 0.001	0.31 (0.85)	0.000 \pm 0.020	0.99 (0.00)
Δ FCR _{R/ETS}	0.000 \pm 0.002	0.96 (0.00)	-0.063 \pm 0.049	0.22 (1.65)

Table S3: Summary of the statistical models (GLMs) testing the relationships between baseline corticosterone as a proxy of chronic stress exposure and (A) oxidative stress parameters at baseline or (B) mitochondrial parameters at baseline. Sex was also included as a fixed factor in the models. Significant parameters ($p \leq 0.05$) are reported in bold, and parameters being marginally significant ($p \leq 0.10$) are reported in italic. Parameters remaining significant after false discovery rate (FDR) correction are underlined and marked with a *. Estimates for sex are given for females.

	Baseline CORT		Sex	
A) Oxidative stress at baseline	Estimate \pm SE	p-value (F)	Estimate \pm SE	p-value (F)
Plasma ROMs	-0.27 \pm 0.62	0.67 (0.19)	3.22 \pm 1.27	0.021 (6.38)
Plasma protein carbonyl	-1.08 \pm 0.51	0.046 (4.56)	-0.19 \pm 1.10	0.86 (0.03)
Plasma 8-OHdG	-1.76 \pm 4.22	0.68 (0.17)	4.27 \pm 8.67	0.63 (0.24)
RBC protein carbonyl	0.13 \pm 0.25	0.61 (0.28)	0.26 \pm 0.52	0.62 (0.26)
RBC 8-OHdG	-3.98 \pm 3.26	0.24 (1.50)	<u>-25.71 \pm 6.68*</u>	0.001 (14.81)
RBC GSSG/GSH	-0.030 \pm 0.011	0.017 (7.16)	0.010 \pm 0.012	0.42 (0.68)
RBC GSSG	0.16 \pm 0.13	0.26 (1.39)	0.03 \pm 0.15	0.85 (0.04)
RBC total GSH	0.64 \pm 0.26	0.030 (6.09)	-0.05 \pm 0.29	0.86 (0.03)
<i>RBC GPx</i>	<i>2.03 \pm 0.99</i>	<i>0.055 (4.19)</i>	1.74 \pm 2.03	0.40 (0.73)
RBC SOD	-108.47 \pm 90.25	0.25 (1.44)	-37.95 \pm 185.23	0.84 (0.04)
RBC OXY	-1.58 \pm 1.33	0.25 (1.43)	-2.59 \pm 2.73	0.35 (0.90)
Plasma GPx	-0.04 \pm 0.04	0.39 (0.76)	0.10 \pm 0.09	0.25 (1.42)
Plasma SOD	-0.84 \pm 0.57	0.15 (2.22)	-2.66 \pm 1.16	0.034 (5.24)
Plasma OXY	-7.25 \pm 4.81	0.15 (2.28)	5.58 \pm 9.86	0.58 (0.32)
B) Mitochondrial parameters at baseline	Estimate \pm SE	p-value (F)	Estimate \pm SE	p-value (F)
ROUTINE	-0.005 \pm 0.022	0.82 (0.06)	0.028 \pm 0.046	0.55 (0.37)
LEAK	0.010 \pm 0.008	0.22 (1.61)	0.024 \pm 0.016	0.15 (2.30)
OXPHOS	-0.015 \pm 0.018	0.41 (0.71)	0.004 \pm 0.037	0.92 (0.01)
ETS	-0.020 \pm 0.040	0.62 (0.25)	0.020 \pm 0.084	0.82 (0.05)
<i>FCR_{L/R}</i>	<i>0.018 \pm 0.009</i>	<i>0.069 (3.73)</i>	0.028 \pm 0.019	0.17 (2.05)
FCR_{L/ETS}	0.021 \pm 0.008	0.024 (6.10)	<i>0.032 \pm 0.017</i>	<i>0.084 (3.34)</i>
FCR _{R/ETS}	0.013 \pm 0.035	0.71 (0.14)	0.034 \pm 0.074	0.65 (0.21)